Carbohydrate Research, 199 (1990) 83–89
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PD: 00-00-1990
P: 83-89 (7)

Effects of reduced malto-oligosaccharides on the thermal stability of pullulanase from *Bacillus acidopullulyticus**

Shuichi Kusano†, Shin-Ichiro Takahashi, Daisaburo Fujimoto, and Yoshiyuki Sakano‡
Laboratory of Biological Chemistry, Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo-183 (Japan)

(Received July 25th, 1989; accepted for publication in revised form, October 15th, 1989)

ABSTRACT

We investigated the effects of the reduced malto-oligosaccharides, D-glucitol (G_1-OH), maltitol (G_2-OH), maltotriitol (G_3-OH), maltotetraitol (G_4-OH), and maltopentaitol (G_5-OH) on the thermal stability of *Bacillus acidopullulyticus* pullulanase (EC 3.2.1.41). The thermal stability depended on the concentration of D-glucitol; after heat treatment for 90 min at 60° in the presence of 0.56, 0.28, 0.14, or 0M G_1-OH, the residual activity was 100, 80, 32, and 10% of the control, respectively. Stability increased with the number of glucosyl residues in the alditols added; the effects of G_3-OH, G_4-OH, and G_5-OH on stability were remarkable. Addition of 30% G_2-OH, G_3-OH, and G_4-OH also contributed to the thermal stability of the pullulanase immobilized onto chitosan beads treated with glutaraldehyde. A high concentration of G_2-OH stabilized other debranching amylases, *Klebsiella pneumoniae* pullulanse, *Bacillus sectorramus* pullulanase, and *Pseudomonas amyloderamosa* isoamylase (EC 3.2.1.68) under heat treatment for 48 h at 60°, as well as the pullullanase of *B. acidopullulyticus*.

INTRODUCTION**

Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase)[1–8] is a debranching amylase that hydrolyzes the (1→6)α-D-glucosidic linkages in pullulan and amylopectin. Another debranching enzyme is isoamylase (EC 3.2.1.68, glycogen 6-glucanohydrolase)[9–12], which hydrolyzes α-(1→6) linkages in glycogen and amylopectin but does not hydrolyze those in pullulan. These debranching amylases have been found in many kinds of microbes and plants. They improve the saccharification of starch to produce glucose, maltose, and malto-oligosaccharides using glucoamylase (EC 3.2.1.3), beta-amylase (EC 3.2.1.2), or alpha-amylase (EC 3.2.1.1). *Bacillus acidopullulyticus* pullulanase (BA-Pase), being thermostable and acidophilic, is generally used in the industrial production of glucose syrup[13,14].

*Supported, in part, by a Grant-in-Aid for Scientific Research (01860016) from the Ministry of Education, Science and Culture of Japan.
† Present address: Ohmiya Research Laboratory, Nikken Chemicals, Co. Ltd., Ohmiya, Saitama-300, Japan.
‡ To whom inquiries should be addressed.
**Abbreviations: SDS, sodium dodecylsulfate; PAGE, poly(acrylamide) gel electrophoresis; SDS–PAGE, poly(acrylamide) gel electrophoresis in the presence of SDS; G_1, G_2, ..., G_5, D-glucose, maltose, ..., maltopentaose; G_1-OH, G_2-OH, ..., G_5-OH, D-glucitol, maltitol ..., maltopentaitol.

0008-6215/90/$ 03.50

We reported that BA-Pase efficiently synthesized α-maltosyl-(1→6)-cyclodextrins from maltose and cyclodextrins[15], and the activities of immobilized BA-Pase columns showed[16] little decrease after continuous operation for 72 days at 60°. In the saccharification of starch, the syntheses of branched cyclodextrins, and other enzymic reactions, the thermal stability of the enzyme is very important; its high stability allows increased reaction temperature and substrate concentration, preventing microbial growth. The thermal stability of purified BA-Pase was enhanced in the presence of 30% (1.6M) D-glucitol[17].

In this paper we describe in detail the effect of substrate analogues, reduced malto-oligosaccharides, on the thermal stability of the pullulanase, and compare them with those of other debranching amylases, *Klebsiella pneumoniae* pullulanase (KP-Pase)[5], *Bacillus sectorramus* pullulanase (BS-Pase)[18], and *Pseudomonas amyloderamosa* isoamylase (PA-Iase)[10].

MATERIALS AND METHODS

Materials. — BA-Pase was purified as described previously[17], and the F-1 fraction (88 units·mg^{-1}), showing a single band on PAGE and SDS-PAGE, was used in this experiment. Crystalline preparations of KP-Pase (32 units.mg^{-1}) and PA-Iase (24 units.mg^{-1}), showing a single band on PAGE and SDS-PAGE, were purchased from Hayashibara Biochemical Laboratories, Inc. BS-Pase was obtained from Amano Pharmaceutical Co., Ltd. and further partially purified using ammonium sulfate precipitation (50–60% saturation) before use. Partially purified BS-Pase (63 units.mg^{-1}) showed a main, active band, with two minor bands (<5%, as checked by protein staining). BA-Pase immobilized onto chitosan beads treated with glutaraldehyde (GA-CB-Pase) was prepared as described previously[16].

D-Glucose (G_1) and maltose (G_2) were purchased from Kokusan Kagaku Co., Ltd. Maltotri- (G_3), tetra- (G_4), and pentaose (G_5) were purchased from Nihon Shokuhin Kakou Co., Ltd. Malto-oligosaccharides were reduced to the corresponding alditols with $NaBH_4$ as follows: 50 mmol of malto-oligosaccharide and $NaBH_4$ (0.3 mol) were dissolved in a final volume of 200 mL and kept for 2 h at room temperature. Mixtures were made neutral with M acetic acid, desalted with Amberlite IRA-411 (OH^- form) and IR-120B (H^+ form), concentrated to syrups, and dehydrated by repeated addition of methanol, followed by evaporation *in vacuo*. The alditols showed negligible reducing power by the Somogyi–Nelson assay[19]. All other reagents were of analytical grade.

Assay of enzyme activity. — PA-Iase (50 μL) was incubated with 200 μL of 0.4% soluble glutinous rice-starch dissolved in 20mM acetate buffer (pH 3.5) for 10 min at 40°C. Each pullulanase preparation (50 μL) was incubated for 10 min with 200 μL of 0.25% pullulan dissolved in 20 mM acetate buffer (pH 5.0) at 40° (for KP-Pase and BS-Pase) or 60° (BA-Pase). The reducing sugar released from each enzyme reaction-mixture was determined by the Somogyi–Nelson method[19]. One unit of individual enzyme activity was defined as the amount of enzyme that catalyzed the hydrolysis of 1 μmol of glycosidic linkage per min. Each enzyme solution (0.02–5 units) was incubated

with a reduced malto-oligosaccharide (0–2.1 mmol) in 50–1200 μL of 50mM acetate buffer (pH 4.5 for PA-Iase, pH 5.0 for BA-Pase and BS-Pase, pH 5.5 for KP-Pase) for 0–48 h at 60°. Mixtures were cooled in a cold bath (4°) for >20 min, and then the residual activity was determined as already described.

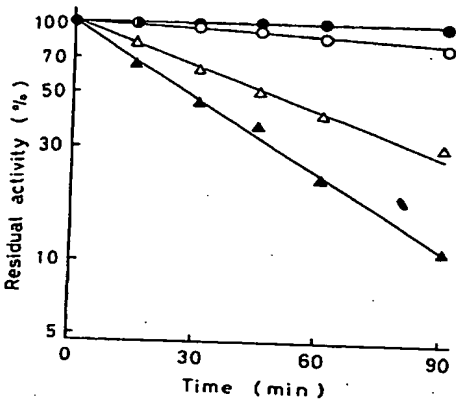

Fig. 1. Effect of D-glucitol concentration on thermal stability of pullulanase. Pullulanase was incubated with 0 M (-▲-), 0.14M (-△-), 0.27M (-○-),and 0.55M (-●-) of D-glucitol at 60°, and then the residual activity was measured as described in Materials and Methods.

RESULTS AND DISCUSSION

Effects of reduced malto-oligosaccharides on the thermal stability of pullulanase. — Figure 1 shows a pseudo-first-order plot for the inactivation of BA-Pase at 60° (k_o = 4.3 x 10^{-4} sec^{-1}). Its thermal stability was greatly enhanced in the presence of D-glucitol (G_1-OH). After heat treatment for 90 min at 60° in the absence of G_1-OH, the residual activity was decreased to ~10% of the control, whereas in the presence of 0.55 M G_1-OH, the enzyme activity decreased little. This effect depended on the concentration of G_1-OH. The effects of G_2-OH and G_3-OH on the thermal stability of this enzyme were better than that of G_1-OH, and also depended on the concentration of these alditols (data not shown). There are many reports of a substrate[20] or Ca^{2+} ion[21–24], promoting the thermal stability of amylases, but we have found few reports concerning the effect of substrate analogues on their thermal stability. On the other hand, Gekko and Idota[25], Gekko and Morikawa[26], and Uedaira and Uedaira[27] reported that some sugars and polyols stabilized the structure of ribonuclease A, chymotrypsin, bovine serum albumin, and lysozyme through strenghtening of the hydrophobic interaction.

The effects of the length of glucose residues in reduced malto-oligosaccharides on the thermal stability of the pullulanase are shown in Fig. 2. After heat treatment for 2 h at 60° without any reduced malto-oligosaccharide present, the residual activity decreased to ~10% of the control, whereas after 2-h heat treatment in the presence of G_1-OH and G_2-OH (0.2M) the activities were 45 and 65%, respectively, and in the

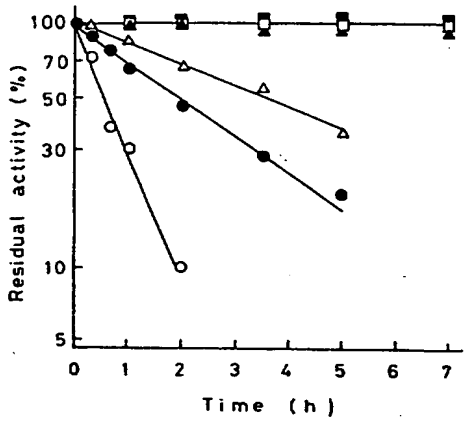

Fig. 2. Effect of reduced malto-oligosaccharides on thermal stability of pullulanase. Pullulanase was incubated at 60° with or without 0.2M reduced malto-oligosaccharide and then the residual activity was measured as described in Materials and Methods. -○-, none; -●-G_1-OH; -△-, G_2-OH; -▲-, G_3-OH; -□-, G_4-OH; -■-, G_5-OH.

presence of G_3-OH, G_4-OH, and G_5-OH (0.2M) the pullulanase activity decreased little until 7 h.

The foregoing results indicate that the thermal stability of the enzyme increased in proportion to the chain length of glucose residues in the alditols added, and that reduced malto-oligosaccharides larger than G_3-OH completely protected the enzyme activity from thermal treatment, probably stabilizing the tertiary structure of the enzyme through a specific interaction of substrate analogues and the enzyme, as well as hydrophobic interaction as already described[25-27].

Effects of reduced malto-oligosaccharides on pullulanase activity. — After the enzyme (0.02 units) had been preincubated with a reduced malto-oligosaccharide (0–2.5 μmol) in a final volume of 50 μL for 5 min at 25° (pH 5.0), 200 μL of 0.25% pullulan (pH 5.0) was added to the mixture, and then the residual activity was determined as described in Materials and Methods. D-Glucitol (G_1-OH) (50mM) did not inhibit the pullulanase activity, but G_2-OH did inhibit it a little. With G_3-OH, G_4-OH, and G_5-OH, the activity rapidly increased in comparison to G_2-OH (Fig. 3).

These results suggest that the inhibition of pullulanase activity by the added alditols, probably surrounding the active site of the pullulanase, was closely related to the effects of these saccharides on the enzyme's thermal stability.

Effects of high concentration of maltitol on the thermal stabilities of debranching amylases. — Under a high concentration of G_2-OH, the effect of temperature on the stabilities of BA- and KP-Pases was investigated. Each enzyme (3 units) was incubated with or without 1.7M G_2-OH in 500 μL of 50mM acetate buffer (pH 5.0 for BA-Pase; pH 5.5 for KP-Pase) at different temperatures (0–95°) for 30 min, diluted 10-fold with the same buffer, and then the residual activity of each enzyme treated was determined as described in Materials and Methods. BA-Pase without G_2-OH was unstable above 55°,

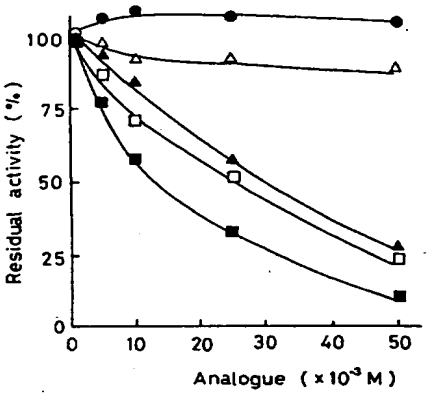

Fig. 3. Effect of reduced malto-oligosaccharides on pullulanase activity. Pullulanase activity was measured in the presence of G_1-OH (-●-), G_2-OH (-△-), G_3-OH (-▲-), G_4-OH (-□-), and G_5-OH (-■-) as desribed in Materials and Methods.

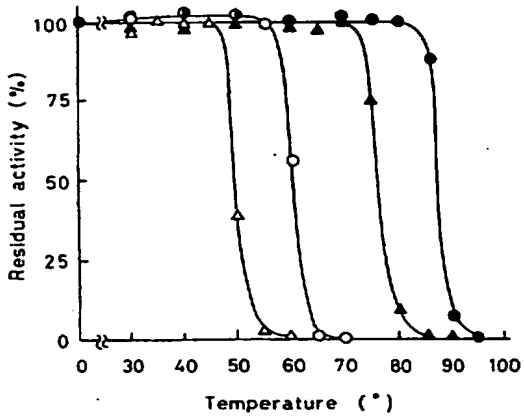

Fig. 4. Effect of 1.7M maltitol on thermal stability of pullulanase. Pullulanase was incubated with or without 1.7M G_2-OH for 30 min and the residual activity was measured as described in Materials and Methods. -○-, BA-Pase; -●-, BA-Pase + G_2-OH; -△-, KP-Pase; -▲-, KP-Pase + G_2-OH.

whereas the residual activity was 86% of the control after treatment for 30 min at 85° in the presence of G_2-OH (Fig. 4). Also, KP-Pase without G_2-OH was unstable above 45°, whereas the activity did not decrease below 70° in the presence of G_2-OH (Fig. 4). With the addition of G_2-OH (1.7M), the LD_{50} of KP-Pase for thermal stability rose from 50 to 76° and that of BA-Pase rose from 60 to 86°. Thermo-unstable KP-Pase and thermostable BA-Pase were found to be more stable under conditions of high-G_2-OH concentration.

The effects of 60°-treatment on the stabilities of four debranching amylases, BA-Pase, BS-Pase, KP-Pase, and PA-Iase, with or without 1.7M G_2-OH were pursued for 48 h and compared (Fig. 5). Without the analogue, the activities decreased rapidly to

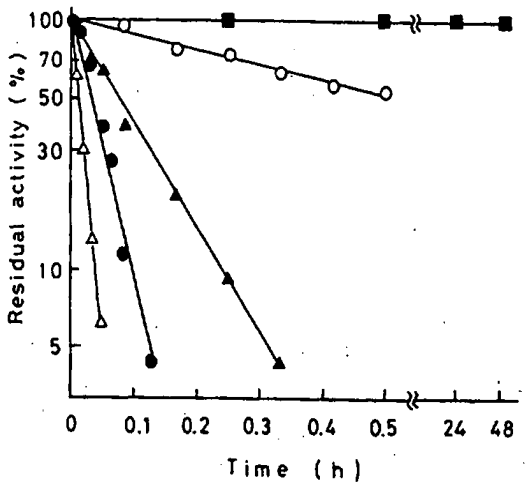

Fig. 5. Effect of 1.7M maltitol on thermal stability of debranching amylases. Each debranching amylase was incubated with 1.7M G_2-OH at 60°, and then the residual activity was measured as described in Materials and Methods. -△-, KP-Pase; -●-, PA-Iase; -▲-, BS-Pase; -○-, BA-Pase; -■-, each debranching amylase + G_2-OH.

0–50% of the control within 30 min; the rate constants of the pseudo-first-order reaction for the inactiviation of BA-Pase, BS-Pase, KP-Pase, and PA-Iase were calculated to be 3.8×10^{-4}, 2.7×10^{-3}, 1.6×10^{-2}, and 6.0×10^{-3} sec^{-1} from the curve in Fig. 5. In contrast, in the presence of 1.7M G_2-OH, the activities of these four debranching amylases decreased little until 48 h; the high-G_2-OH concentration protected them, containing thermo-unstable enzymes, from the thermal effect and stabilized their tertiary structure. Used industrially with a high concentration of substrate in conjunction with other enzymes, the thermal stability of these enzymes makes them suitable for the production of glucose, maltose, and other compounds from starch.

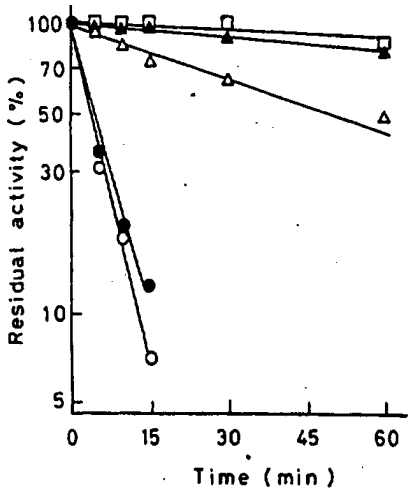

Fig. 6. Effect of reduced malto-oligosaccharides on the thermal activity of immobilized pullulanase. Immobilized pullulanase was incubated at 60° with or without 30% reduced malto-oligosaccharides and then residual activity was measured as described in Materials and Methods. -○-, none; -●-, G_1-OH; -△-, G_2-OH; -▲-, G_3-OH; -□-, G_4-OH.

Effects of reduced malto-oligosaccharides on the thermal stability of immobilized pullulanase. — The effect of the alditols (from G_1-OH to G_5-OH) on the thermal stability of an immobilized pullulanase, GA-CB-Pase, depended on their concentration (data not shown), in a manner similar to that of the free pullulanase already described. This suggests that the high stability of the immobilized enzyme, having a long half-life (after continuous reaction for 72 days at 60°, its activity had scarcely decreased[16]), was attributable to the high concentration of the substrate (more than 1.4M).

As shown in Fig. 6, the chain length of glucose residues in the alditols affected the thermal stability of GA-CB-Pase. These effects increased in proportion to length, as was true with the free pullulanase already described.

REFERENCES

1 H. Bender and K. Wallenfels, *Biochem. Z.*, 334 (1961) 79–95.
2 S. Ueda and H. Nanri, *Appl. Microbiol.*, 15 (1967) 492–496.
3 G. J. Walker, *Biochem. J.*, 108 (1968) 33–40.
4 D. J. Manners and K. L. Rowe, *Carbohydr. Res.*, 9 (1969) 107–121.
5 M. Abdullah and D. French, *Arch. Biochem. Biophys.*, 137 (1970) 483–483.
6 E. Y. C. Lee, J. J. Marshall, and W. J. Whelan, *Arch. Biochem. Biophys.*, 143 (1971) 365–374.
7 Y. Takasaki, *Agric. Biol. Chem.*, 40 (1976) 1515–1522.
8 M. Abdullah, B. J. Catley, E. Y. C. Lee, J. F. Robyt, K. Wallenfels, and W. J. Whelan, *Cereal Chem.*, 43 (1966) 111–118.
9 Y. Sakano, T. Kobayashi, and Y. Kosugi, *Agric. Biol. Chem.*, 33 (1969) 1535–1540.
10 K. Yokobayashi, A. Misaki and T. Harada, *Biochim. Biophys. Acta*, 212, (1970) 458–469.
11 R. M. Evans, D. J. Manners, and J. R. Stark, *Carbohydr. Res.*, 76 (1979) 203–213.
12 Z. Gunja-Smith, J. J. Marshall, E. E. Smith, and W. J. Whelan, *FEBS Lett.*, 12 (1970) 96–100.
13 B. E. Norman, *Denpun Kagaku*, 30 (1983) 200–211.
14 B. F. Jensen and B. E. Norman, *Process Biochem.*, 19 (1984) 129–134.
15 Y. Sakano, M. Sano, and T. Kobayashi, *Agric. Biol. Chem.*, 49 (1985) 3391–3398.
16 S. Kusano, T. Shiraishi S.-I. Takahashi, D. Fujimoto, and Y. Sakano, *J. Ferment. Bioeng.*, 68 (1989) 233–237.
17 S. Kusano, N. Nagahata, S.-I. Takahashi, D. Fujimoto, and Y. Sakano, *Agric. Biol. Chem.*, 52 (1988) 2293–2298.
18 K. Tsuji, M. Shiosaka, S. Hirose, K. Nakai, N. Yokoi, and R. Ohya, *Nippon Nogeikagaku Kaishi*, 63 (1989) 402 (in Japanese).
19 M. Somogyi, *J. Biol. Chem.*, 195 (1952) 19–23.
20 H. H. Hyun and J. G. Zeikus, *Appl. Environ. Microbiol.*, 49 (1985) 1168–1173.
21 J. A. Thoma, J. E. Spradlin, and S. Dygert, *Enzymes*, 5 (1971) 115–189.
22 T. Takagi, H. Toda, and T. Isemura, *Enzymes*, 5 (1971) 235–271.
23 N. Nakamura, N. Sashihara, H. Nagayama, and K. Horikoshi, *Denpun Kagaku*, 34, (1987) 38–44.
24 A. R. Plant, H. W. Morgan, and R. M. Daniel, *Enzyme Microb. Technol.*, 8 (1986) 668–672.
25 K. Gekko and Y. Idota, *Agric. Biol. Chem.*, 53 (1989) 89–95.
26 K. Gekko and T. Morikawa, *J. Biochem. (Tokyo)*, 90 (1981) 39–50.
27 H. Uedaira and H. Uedaira, *Bull. Chem. Soc. Jpn.*, 53 (1980) 241–2455.